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Molecular biology and gene therapy in the treatment of chronic pain

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In the last two decades, advances in the fields of gene transfer and cellular grafting have rendered application of these technologies to the nervous system practical. The potential for cell-based therapies has benefited from new concepts in the means for cell delivery as well as a broadened repertoire of potential cells for grafting. Both developments create the opportunity for increased control of graft behavior and safety of application. Similarly, gene transfer strategies have evolved such that potentially therapeutic transgene expression lasts longer and poses less of a risk to host tissue. Finally, the fusion of these technologies raises the potential for enhanced control of graft behavior as well as enhanced delivery of the therapeutic proteins encoded by gene transfer.

Current pain therapies are encumbered by a variety of limitations. Systemic pharmacologic manipulation of pain is plagued by addiction and tolerance as well by as the secondary effects of these drugs on the patients' level of consciousness and emotional status. Surgical interventions for medically refractory pain also possess inherent limitations. Both neuroablation and neuroaugmentation are discussed in other articles in this issue. The former has the inherent limitation of being destructive to the nervous system, whereas the latter requires implanted hardware that is susceptible to the vicissitudes of infection, breakage, and malfunction.

The nervous system takes advantage of both anatomic and pharmacologic specificity to produce the substrate for experience and behavior. Pharmacologic intervention can achieve increased functional specificity through the refinement of selective receptor affinity. Because the nervous system may use the same receptors in the synaptic machinery of different functions, however, selective ligand affinity may never achieve functional specificity. Similarly, the surgical approaches of ablation and neural stimulation provide anatomic specificity without pharmacologic specificity. Multiple receptor-based systems can serve different functions within the same anatomic locations in addition to serving various functions within a single synapse. The latter can be observed in synapses that use both monoaminergic and peptide neurotransmitters. Thus, approaches that can achieve both pharmacologic and anatomic specificity have an inherently greater potential to achieve functional specificity. In one example, attempts to deal with intractable segmental pain syndromes, such as herpetic neuralgia, would benefit from treatment strategies capable of altering the synaptic function of a limited set of dorsal horn and dorsal root ganglia (DRG) neurons with respect to the activity of specific transmitter systems. Gene transfer within the nervous system and cellular grafting strategies may provide this dual advantage.

Nonetheless, cellular and molecular therapies for the nervous system remain in their infancy. The present article reviews the experiments that provide

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the proof of principle for the concepts described. It is also important to describe the limitations of these approaches. Human gene transfer experiments have met with their first documented clinical successes at the same time that a variety of complications of these therapies have been recognized. These complications underscore the importance of circumspection in proceeding with cellular and molecular therapies, particularly for the treatment of chronic syndromes, such as pain.

Introduction to gene transfer

The best method of gene transfer remains a topic of debate. Gene delivery is initiated when a vector binds to the host cell. If entry into the cell occurs through endocytosis, the endosome fuses with a lysosome and leads to enzymatic degradation of the transgene. Therefore, an effective delivery method must either avoid entry into the cell via endocytosis, thus preventing lysosomal degradation, or allow transgene entry into the cell through an alternative pathway. For most transgenes to be expressed, they must enter the nucleus. The nuclear membrane serves as a relatively effective barrier against the entry of foreign nucleic acid [1]. Early in the development of gene therapy, entry into the nucleus was limited to mitotically active cells. Because the nuclear membrane functions as a barrier, successful nuclear entry required the natural breakdown of the nuclear membrane [2–4]. Recent advances have involved the use of nuclear localization signals and viral vectors to overcome this barrier. The final and most important step of gene transfer is expression of the transgene. The duration of expression can vary from transient to extended depending on the vector being used. Long-term expression is the obvious goal of gene therapy and is usually accomplished through integration of the transgene into the host DNA. Different vector systems use a variety of pathways to achieve integration, which involves several steps (Fig. 1).

In addition to these characteristics, an ideal vector should not be a source of pathogenicity to the host cell. Thus, an ideal vector is nontoxic and elicits little if any immune response in the host. Vectors for gene therapy can be divided into nonviral and viral groups.

Nonviral gene therapy

The major advantage of nonviral approaches to gene therapy is their lack of pathogenicity. Delivery of naked DNA is the simplest form of nonviral vector gene therapy. In 1980, Capecchi [1] was able to successfully microinject DNA via glass micropipettes into the nuclei of host cells in vitro. In 1990, Wolff and colleagues [5] showed that DNA and RNA transgenes could be effectively expressed when injected into mouse skeletal muscle. Injection into other major organs, such as the liver, spleen, and brain, resulted in relatively inefficient transgene expression, however [5]. To increase the efficiency of gene delivery with naked DNA, researchers have developed novel delivery methods. One method of delivery uses electropermeabilization to enhance the uptake of plasmid DNA. Lin et al [6] designed an intrathecal electroporation probe that greatly enhanced uptake in the spinal cord when used after intrathecal injection of plasmid DNA. Although uptake was enhanced, expression of the transgene remained transient and greatly diminished after 2 weeks. The experiment used intrathecal electroporation with a range of voltages from 50 V up to 250 V, administered over time periods varying from 25 to 100 ms pulses in order to boost uptake of a plasmid-encoding green fluorescent protein (GFP). GFP expression was detected in the spinal cord meninges and superficial layers of the spinal cord. Gene expression peaked between 3 and 7 days and diminished by day 14. Neither deleterious behavioral or histologic changes were detected in conjunction with gene expression.

To increase the uptake of DNA into the host cell, plasmid DNA has been combined with nonviral carriers. One method used by Yang et al [7] involved coating fine gold particles with the

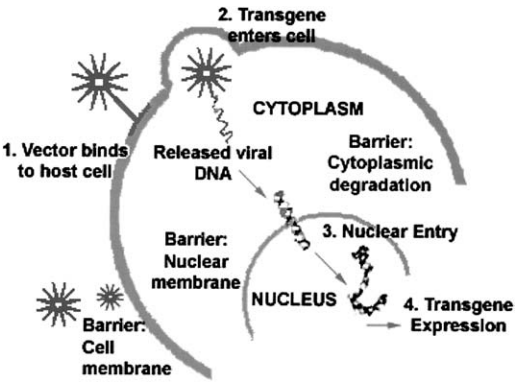

Fig. 1. Steps required for gene delivery. Four basic steps are required for effective gene delivery leading to successful transgene expression. The cellular and nuclear membrane barriers must be penetrated, and cytoplasmic degradation must be avoided. (See also Color Plate 1.)

transgene. In vivo particle bombardment effectively transferred genes to major organs in both rats and mice. Another method of delivery, known as liposome-mediated transfer, involved combining plasmid DNA with cationic lipids to form lipoplexes [8]. Liposome-mediated DNA transfer was successfully used in clinical trials for the delivery of the cystic fibrosis transmembrane conductance regulator (CFTR) to nasal epithelium [9]. Different variants of lipoplexes have been devised. Lipopexes with cationic polymers, such as poly L-lysine or protamine, have improved uptake and expression for a variety of reasons. First, the polymers are able to condense the size of the lipoplex while producing an excess of cationic charges. This coating protects the transgene from nuclease activity after endocytosis. In addition, the supercoiled conformation of the transgene remains intact. Poly L-lysine coating resembles a nuclear localization signal (NLS), thus enhancing transgene entry into the host cell nucleus [10]. Dioleoyl-phosphatidylethanolamine (DOPE) is another common addition to lipoplexes. DOPE is a neutral lipid that facilitates membrane fusion and is capable of destabilizing the lysosomal membrane. Destabilization of the lysosomal membrane allows for release of the plasmid into the host cell cytoplasm, thus reducing the likelihood of lysosomal transgene degradation [11,12].

Cationic polymers provide an alternative to cationic lipids for the delivery of plasmid DNA because they are effective at condensing DNA. Polyethylenimine (PEI) is an example of a polymer currently being used (Fig. 2). In addition to its DNA condensing properties, PEI acts as a proton sponge, causing osmotic disruption of the lysosome. Protection of the delivered gene allows for greater transfection efficiency [13].

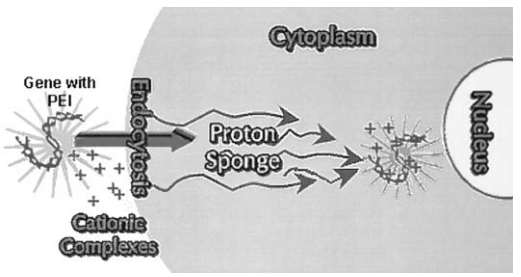

Fig. 2. Schematic representation of polyethylenimine (PEI)-enhanced delivery. Cationic polymers, such as PEI, provide an alternative method of enhanced DNA plasmid delivery in gene therapy. Their ability to condense plasmid DNA and cause lysosomal osmotic disruption contributes to increased transfection efficiency. (See also Color Plate 2.)

Transposons have also emerged as an effective method of delivering plasmid DNA into the host cell. Transposons are naturally occurring elements capable of integrating foreign plasmids into the host cell DNA with the help of two enzymes, integrase and transposase. Integration allows for long-term transgene expression. "Sleeping Beauty" (SB) is one such transposon. The SB transposon was first used to deliver human coagulation factor IX in hemophilia B mouse models. Factor IX was present in therapeutic levels for longer than 5 months, demonstrating the ability of transposons to allow for long-term gene expression [14].

Viral vectors

Unlike nonviral systems, viral vectors have the potential to precipitate an immune reaction. Also, the transgene size that can be delivered by most viral vectors is limited. Nonetheless, the overall efficiency of viral vector gene transfer compared with nonviral vectors has led researchers to develop strategies that may decrease the limitations of viral vectors while maintaining their effectiveness.

Several viral vector systems have been used for gene transfer in vivo, including adenovirus, herpes simplex virus (HSV), recombinant adenoassociated virus (rAAV), and lentiviruses. The existence of standard techniques for gene insertion and deletion allows for viral attenuation or the prevention of viral replication as well as for the addition of potentially therapeutic genes [15]. Vectors constructed with adenovirus [16], HSV [17,18], AAV [19], and lentivirus [20] can effectively transduce neurons, suggesting that these vectors may be applied to the treatment of neurologic disorders.

Fig. 3 illustrates the process of converting a wild-type virus into a useful vector for gene transfer. Transgene expression is accomplished through the construction of an expression cassette. At its heart, an expression cassette possesses three elements: a promoter, a transgene, and a polyadenylation sequence. The promoter drives the expression of the transgene. The polyadenylation sequence attached at the 3′ end of the transgene is necessary for the translation of the resulting mRNA in eukaryotic cells. The expression cassette must be packaged in a viral coat. Packaging can be accomplished by inserting the expression cassette into the wild-type viral genome. Viral coats have a limited capacity to

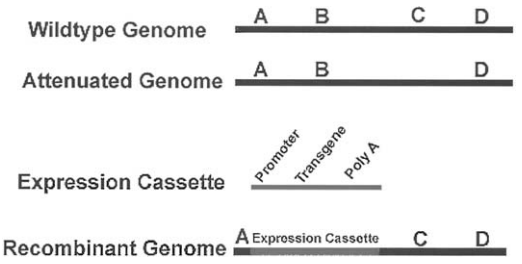

Fig. 3. Components necessary for production of a viral vector. Illustration of the mechanisms involved in the conversion of a wild-type virus into a vector for gene transfer. Key components include the promoter, transgene, and polyadenylation (PolyA) sequence in the expression cassette. (See also Color Plate 3.)

package DNA, however. Therefore, some sequence from the wild-type genome must be removed to make room for the expression cassette. In addition to delivering the expression cassette, the viral vector must be prevented from reproducing and hence causing a myelitis or cerebritis. *Attenuation* is the term used to describe rendering the virus incapable of reproducing. Elements of the viral genome necessary for replication are deleted, hence creating the necessary room for insertion of the expression cassette. The result is an attenuated virus capable of gene transfer.

A variety of promoters have been applied to the control of potentially therapeutic genes. The Rous sarcoma virus (RSV) and cytomegalovirus (CMV) promoters are the most commonly applied promoters. They drive high-level gene expression in a broad range of cell types, including cells of the nervous system. Cell type–specific promoters have also been applied extensively. The most common examples of cell type–specific promoters applied to the nervous system include the neuron specific enolase (NSE) and glial fibrillary acidic protein (GFAP) promoters. These promoters function selectively in neurons and glia, respectively [21]. Thus, selective gene expression can be achieved through variation of the promoter or selection of vectors that possess selective tropism for different cells. Finally, promoters may be altered to achieve control of gene expression. This latter factor plays a critical role in gene-based neuromodulation, which is discussed in greater detail later in this article.

As previously mentioned, the use of implantable devices for drug delivery and electric stimulation has the disadvantage of potential device malfunction and foreign body infection. Nonetheless, they possess the advantage of offering exquisite control. It is precisely this controllability that is at the heart of the replacement of ablative procedures by device-based neural augmentation. For gene-based approaches to provide an alternative to device-based neural augmentation, similar control must be achieved. Control of gene expression has had a variety of proposed solutions, including the use of inducible and repressible promoters. The most common examples of these are the tetracycline (tet) inducible and tet repressible promoters [22,23]. With these promoters, the application of tetracycline amplifies or suppresses gene expression. Unfortunately, the performance of the tet inducible and tet repressible promoters in vivo has proved disappointing. The newer "tet-on" and "tet-off" promoter systems seem to have significantly augmented capacity for control of transgene expression in vivo [24]. Another approach to expression control involves the placement of elements within the expression cassette that allow for selective removal of the critical elements for gene expression. The Cre-Lox system is one example of this approach [25]. Cre is a recombinase that recognizes an oligonucleotide sequence called LoxP. It is possible to create an expression cassette that contains the Cre gene under the control of an inducible promoter in conjunction with the remaining expression cassette driving expression of the therapeutic transgene. If this latter element contains the LoxP sequence, inducing Cre expression permanently turns off the expression of the therapeutic transgene.

Adenovirus

To date, most work in the nervous system has been performed with adenoviruses. One example of such activity is that of Davidson and colleagues [26], who used an adenoviral vector carrying the hypoxanthine-guanine phosphoribosyltransferase (HGPRT) gene to correct enzyme deficits in a mouse model of Lesch-Nyhan syndrome. Adenoviral vectors have also been used to deliver neurotrophic factors to spinal motor neurons. Using an axotomy model for progressive motor neuron loss, two groups successfully attenuated cell loss with brain-derived neurotrophic factor (BDNF), ciliary neurotrophic factor (CNTF), and glial-derived neurotrophic factor (GDNF). In each case, the growth factors were delivered to the injured motor neurons via retrograde transport after intramuscular injection of the appropriate adenoviral vector [27–29]. Adenoviral gene transfer to sensory neurons of the dorsal horn has

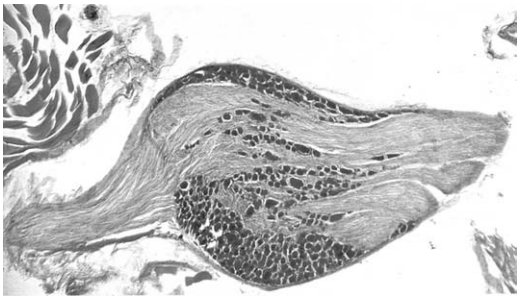

Fig. 4. Retrograde axonal transport. Tissue demonstrating positive adenoviral gene transfer via retrograde axonal transport to dorsal root ganglia primary sensory neurons. (See also Color Plate 4.)

been accomplished via direct injection into the spinal cord [30,31]. Adenoviral gene transfer to dorsal horn and DRG neurons has also been achieved via retrograde transport (Fig. 4) [32,33]. Transgene delivery to sensory neurons via axonal transport of vectors can be achieved through intramuscular, subcutaneous, or peripheral nerve injection. Delivery of neuromodulatory transgenes in this fashion has the potential to alter pain transmission in individual segments without eliminating baseline sensation. Appropriate transgene candidates are discussed later in this article.

One restriction of adenoviral vectors is the limited 3-week time course of expression [19,30–34]. Termination of gene expression may be dependent on cytolytic or noncytolytic mechanisms. Cytolytic termination of gene expression could result from direct viral toxicity [35] or the action of immune mediators [36]. The immune response to viral vectors is biphasic, with an early response to the viral capsid proteins. This phase of the reaction involves activation of microglia, CD4+ T cells, and macrophages [37]. In a later phase of the inflammatory response, CD8+ T cells respond to viral proteins presented to major histocompatibility complex (MHC) class I molecules in transduced neural cells [38]. Remote delivery is likely to limit the early phase of the inflammatory response in the central nervous system (CNS) by eliminating viral capsid proteins from this region. Leaky expression of viral proteins, even from replication-defective vectors, could still trigger a late-phase CD8+ response, however, resulting in neural cell death. Nonetheless, neuron survival after in vivo gene transfer in the remote delivery paradigm has been established [39]. This finding suggests that a noncytolytic mechanism is responsible for the termination of gene expression. Because immunosuppression prolongs gene expression, it is suspected that inflammatory cytokines terminate gene expression at the level of promoter regulation.

The recent work of Thomas et al [40] suggests that the early-stage immune response to CNS injection of first-generation adenoviral vectors is limited in duration and is functionally insignificant. The later stage of the inflammatory response within the nervous system only occurs when the immune system has been sensitized to adenoviral gene products through expression in non-CNS environments. Thus, prolonged expression of first-generation adenoviral transgenes is possible if vector administration can be confined to the CNS. Advanced generation adenoviral "gutted" vectors, in which all viral sequences are deleted, do not seem to be susceptible to this peripheral sensitization-dependent shutdown. This finding suggests that leaky expression of viral genes is responsible for the termination of CNS expression in first-generation vectors.

Adenoassociated virus

Unlike lentivirus- and herpesvirus-based vector systems, adenoassociated virus (AAV) is a parvovirus that has not been linked to any human pathologic processes [41]. The AAV capsid has no envelope and is 20 to 25 nm in diameter. It contains a 4680-base single-stranded DNA genome [42]. The viral genome integrates into host cell chromosomes. The AAV genome has been detected as a single integrated copy in two experiments [43,44]. Nonetheless, multiple copy integration has been demonstrated, and some authors suggest that the genome can exist in host cells without integration [45]. The integrated AAV remains in a latent state until the host cell is infected with either an adenoviral or herpes helper virus. The presence of genes from the helper virus allows wild-type AAV to enter a lytic cycle [46].

rAAV vectors contain only 4% of the wild-type genome. The remaining DNA consists of the inverted terminal repeat sequences (ITRs) at the 5′ and 3′ ends, which allow for DNA packaging in the viral coat [47]. rAAV can infect a broad range of cells in multiple species, including human, nonhuman primate, canine, murine, and avian cells. In addition, it is capable of transducing both dividing and nondividing cells. This latter characteristic lends rAAV to application in the CNS. rAAV can selectively transduce neurons [48], with gene expression in the spinal cord present up to

1 year after direct injection of type 2 rAAV [49]. Duration of gene expression can vary depending on the promoter used and the specific neuroanatomic location of delivery. Klein et al [50] noted an early loss of expression in the hippocampus of rats injected with an rAAV transgene driven by a CMV promoter. Mandel and colleagues [51], who found that fusion of the CMV promoter with the human β-globin promoter prevented early shutdown of gene expression, demonstrated expression beyond 1 year. The NSE promoter provides a means of targeting gene expression to neurons, with expression observed 19 months after injection [50]. With the NSE promoter, neuronal gene expression is present in different types of neurons, including GABAergic, cholinergic, and dopaminergic cells [50]. In parallel, application of the myelin basic protein promoter enhances gene expression in the white matter above the levels seen with CMV, NSE, or GFAP promoters [19].

Of the six AAV serotypes, the application of AAV type 2 (AAV2) to the nervous system has been studied in the greatest depth and seems to be taken up selectively by neurons. In nonneuronal in vitro systems, the uptake of AAV2 depends on binding to heparin sulfate proteoglycans [52]. CNS injection of AAV2 seems to have no deleterious effects. Chamberlin and colleagues [53] were unable to detect microglial infiltrates or gliosis in transgene-expressing tissue. Additionally, behavioral pharmacology experiments were unable to detect any damage to the substantia nigra function after rAAV injection [19]. Whether rAAV undergoes reliable axonal transport continues to be debated. In our hands and those of other investigators, peripheral nerve injection of rAAV vectors results in transgene expression in both motor and DRG sensory neurons (Fig. 5).

The absence of destructive effects on tissue has laid the groundwork for the therapeutic use of AAV in the nervous system [54]. Two approaches have been proposed for the treatment of Parkinson disease. The first approach involves rAAV-mediated delivery of critical enzymes in the dopamine synthetic pathway, including tyrosine hydroxylase, GTP cyclohydrase, and aromatic amino acid decarboxylase, whereas the second approach involves the delivery of neurotrophic growth factors aimed at preventing nigral degeneration [54]. rAAV has also been applied to other models of CNS injury. Mandel et al [55] demonstrated that type 2 rAAV nerve growth factor (NGF) and BDNF transgene delivery to the basal forebrain prevented the loss of cholinergic neurons induced by fornix lesions. Similarly, the delivery of BDNF to the spinal cord using rAAV protected rubrospinal neurons from atrophying after spinal cord injury [56]. Alternatively, the rAAV-mediated delivery of antisense sequence has the capacity to reduce expression of genes and alter neural functional properties. Xiao et al [57] report that the expression of antisense sequence of the α_1 subunit of the gamma-aminobutyric acid (GABA) receptor in the inferior colliculus reduces GABA ligand binding and increases seizure activity.

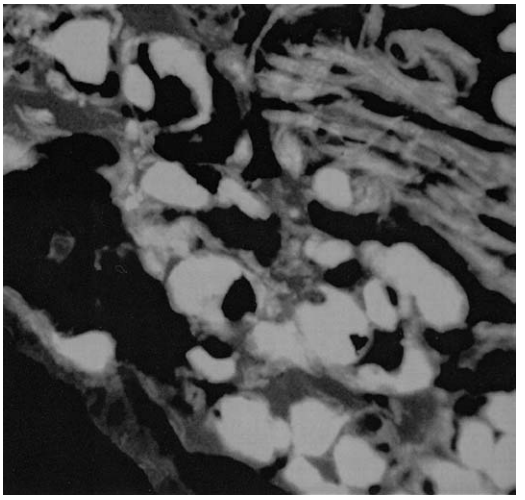

Fig. 5. Green fluorescent protein (GFP) expressing dorsal root ganglia (DRG) neurons. Peripheral nerve injection of adenoassociated virus GFP resulted in DRG expression of GFP. (See also Color Plate 5.)

Herpes simplex virus 1

HSV-1 is another attractive vector for the delivery of therapeutic genes to the nervous system. HSV-1 is able to infect nondividing cells. The HSV-1 genome is composed of 152-kilobase (kb) double-stranded DNA. The relatively large size of the genome allows for insertion of large transgenes and general ease of genetic manipulation. The most distinguishing characteristic of HSV-1 as a viral vector is its ability to establish latent infection in neurons [58].

Like other vectors, to be used as an effective tool for therapy, HSV-1 must be modified to attenuate its virally mediated toxicity. One method of attenuation has been to remove all viral sequences except the viral recognition sequences. These vectors are known as amplicons. The absence of

any genes encoding viral proteins prevents amplicons from delivering any viral genes to the target cell. The absence of these genes makes vector production more difficult, however. Geller and his colleagues [59,60] demonstrated the use of such a defective HSV-1 vector to deliver the *Escherichia coli lacZ* gene to neurons in vitro.

The HSV-1 genome contains three main classes of genes, including immediate early (IE), early (E), and late (L) genes. It was found through mutational analysis that most of these genes were not essential for viral replication in cell cultures. The development of HSV-1 deletion mutants has proven effective for the delivery of reporter genes into postmitotic cells [61]. As mentioned earlier, however, the use of recombinant viruses is risky because of their potential for reverting to wild type. Thus, research continues to identify effective HSV recombinants with the fewest essential genes necessary for infection.

Retroviral vectors

Retroviral vectors have multiple characteristics that are advantageous for gene transfer. First, their ability to integrate transgene expression cassettes into the host chromosome creates stable long-term gene expression. Their ability to transfer expression cassettes without virus-derived sequence reduces the potential for host cell destruction by CD8 lymphocytes. Finally, these vectors can accommodate insertion of sequences as large as 10 kb [62]. Nonetheless, early-generation retroviruses were of little use for neuroprotection because they were incapable of gene transfer in terminally differentiated neurons [63].

Unlike the oncoretroviruses used for early-generation vectors, however, lentiviruses are capable of replication in nonmitotic cells because they possess enzymes that allow for nuclear envelope docking and energy-dependent transport across the nuclear membrane [64]. Using the well-understood genetics of human immunodeficiency virus (HIV) 1, investigators constructed HIV-based retroviral vectors capable of transducing neurons [65]. The similarity of these early lentiviral vectors to wild-type HIV posed the danger of recombination and possible HIV infection.

Third-generation lentiviral vectors are pseudotyped with the coat proteins of non-HIV viruses. In addition, these vectors contain only three of the nine genes of the wild-type virus. Lentiviral vectors contain only the genetic material of the expression cassette and the sequence necessary for its encapsidation, reverse transcription, and integration. Also, because the viral long terminal repeat (LTR) loses its ability to support replication after transfer to the host, the chance of creating progeny competent of replication is further reduced.

Despite these precautions, human application of HIV-derived vectors remains controversial. This concern has motivated the development of vectors based on nonprimate lentiviruses. Feline immunodeficiency virus (FIV) is one such vector. FIV is unable to infect human cells, but when the vesiculostomatitis virus protein (VSVG) is inserted into the viral capsule, transfection of nondividing human cells is possible [66]. Equine infectious anemia virus (EIAV) is another such virus [67] and provides an attractive backbone for a vector system. An EIAV vector holds promise for several reasons. First, its genome is among the simplest of the lentiviruses, rendering it relatively easy to manipulate. Second, it is incapable of replication in human cells, rendering it nonpathogenic in human beings even in its wild-type form. Tropism may be altered in EIAV by inserting foreign proteins into the vector's lipid capsule (pseudotyping), and EIAV is similar to FIV in this respect.

Mazarakis et al [68] demonstrated that Rabies-G pseudotyped lentiviral vectors constructed using this strategy are capable of transducing both striatal and hippocampal neurons in vitro with similar efficiency. In vivo, Rabies-G pseudotyped vectors injected into the striatum were capable of transducing neurons in multiple anatomic locations projecting into this region, suggesting that these vectors undergo retrograde axonal transport. VSVG pseudotyped vectors were incapable of transducing these distant neurons. When VSVG and Rabies-G pseudotyped vectors were injected directly into the spinal cord, the latter vector transduced more neurons, with a wider distribution. This technique yielded gene transfer to 67% of the motor neurons within the transduced region. Neither functional damage nor histologic evidence of inflammation was detected in the spinal cords of these animals. Finally, intramuscular injection of Rabies-G pseudotyped lentivector resulted in transduction of 27% of FluoroGold-labeled neurons, whereas VSVG transduced none. These results suggest that Rabies-G pseudotyped lentivectors may provide another means for remote gene delivery to the DRG and dorsal horn. Unlike adenovirus, however, targeted lentivirus has long-term gene expression and is unlikely to trigger an inflammatory response.

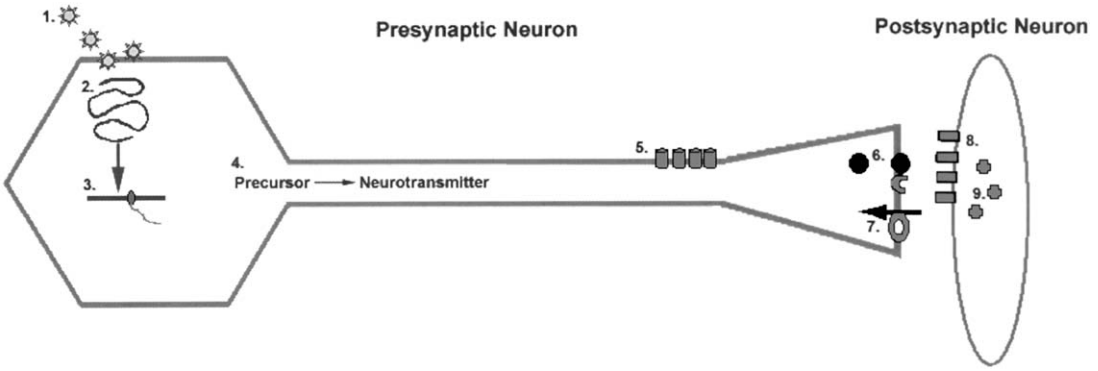

Fig. 6. Gene-based neuromodulation. (*1*) Neuronal vector uptake (*2*) Transgene transcription. (*3*) Transgene translation. (*4*) Neurotransmitter precursor or neurotransmitter synthetic enzyme. (*5*) Ion channel. (*6*) Vesicle docking protein. (*7*) Neurotransmitter reuptake protein. (*8*) Receptor. (*9*) Second messenger. (See also Color Plate 6.)

Gene-based neuromodulation

By inducing the expression of genes that play a role in synaptic activity, the functional role of these neurons may be altered. Fig. 6 illustrates a variety of potential transgenes that can alter synaptic function. Gene-based neuromodulation encompasses the attempt to augment the activity of a specific neurotransmitter system by transferring the gene for the enzymes that produce the neurotransmitter. For example, several authors have pursued the transfer of genes for enzymes that produce dopamine as a means of replacing dopamine production in models of parkinsonism. Combinations of the genes for tyrosine hydroxylase (TH) and aromatic acid decarboxylase (AADC) have successfully enhanced dopamine production and reduced the functional consequences of dopamine depletion in a variety of models [69,70].

The transfer of the gene for the rate-limiting enzyme in GABA production, glutamate decarboxylase (GAD), has been investigated as a means of increasing inhibitory synaptic activity. Several classes of viral vectors, including adenoviral, retroviral, and AAV GAD vectors, have been designed with confirmed GABAergic activity in cell lines [71], primary neuronal and glial cultures [72,73], and organotypic hippocampal cultures [72]. The creators of these GAD vectors postulated that they might act therapeutically in a variety of disorders thought to result from excess synaptic excitation, including neurodegenerative disorders and epilepsy.

More recently, GAD gene transfer has been applied to focused neuromodulation in models of Parkinson disease [74]. Electrophysiologic evidence suggests that subthalamic nucleus (STN) GAD65 gene transfer has the capacity to change the excitatory output of this nucleus to inhibitory output. Additionally, microdialysis confirmed that GAD65 gene transfer increased the production of GABA in the substantia nigra (SN) after STN stimulation [75]. These experiments suggest that GAD65 gene transfer represents a means of focused neural inhibition. Based on this rationale, a phase I trial of GAD gene transfer to the human STN has recently been approved by the US Food and Drug Administration (FDA).

Histologic studies have revealed a high concentration of both GABA and GAD in a variety of hypothalamic nuclei, including the LH [76]. These findings motivated an attempt in one experiment to manipulate feeding behavior through the local administration of GABA antagonists. Injection of bicuculline into the LH results in hyperphagia, suggesting that GABA receptors capable of modulating feeding behavior exist within the LH [77]. Conversely, LH GABA injection results in reduced neuronal firing, and LH GABA and muscimol (GABA agonist) injection reduces food intake [78]. These studies suggest that LH GABA receptors regulate food consumption.

To test the potential of focused gene-based neuromodulation, our laboratory demonstrated that precise LH gene expression could be induced through stereotactic microinjection of rAAV GFP (Fig. 7). The proximity of the LH to more medial structures that drive anorexic behaviors makes precise targeting and distribution of gene transfer requisite to affect focused neuromodulation. Next, the effect of stereotactic LH microinjection of rAAV GAD65 and rAAV GFP on feeding and

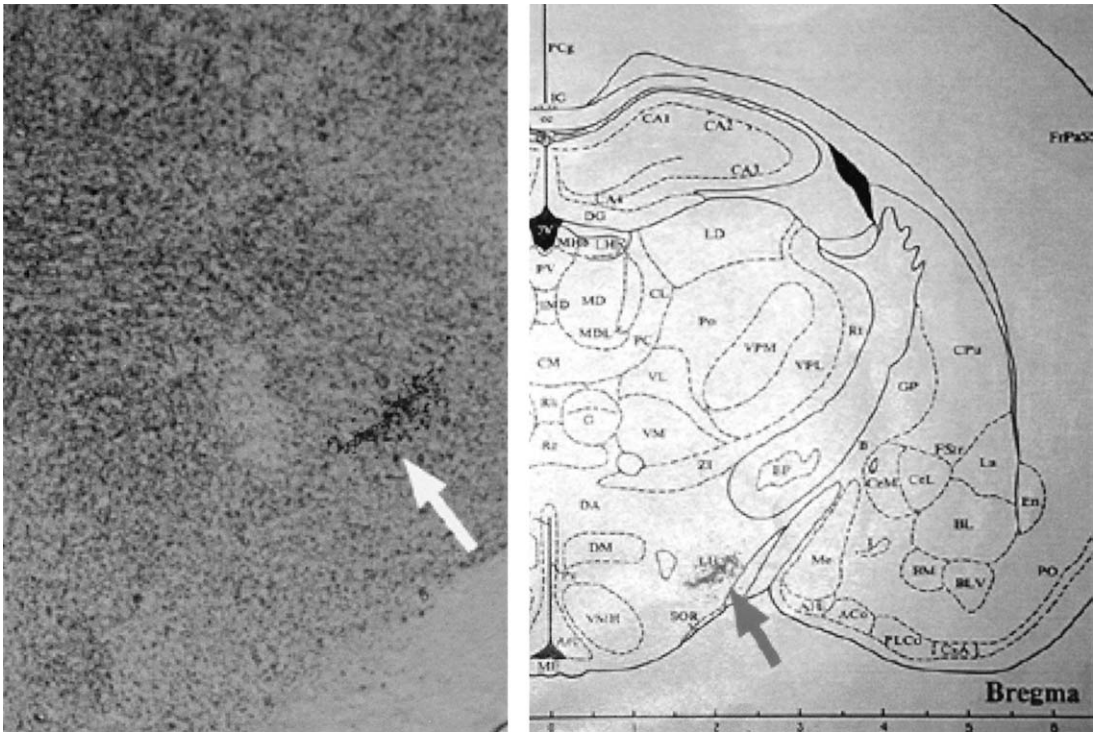

Fig. 7. Green fluorescent protein (GFP) distribution in lateral hypothalamus after stereotactic injection. This figure illustrates positive GFP expression in the lateral hypothalamus after injection of 500 nL of adenoassociated virus glutamate decarboxylase GFP (*left*) and the Paxinos and Watson [103] atlas illustration of the targeted area (*right*). (See also Color Plate 7.)

weight gain were compared, showing that GAD65 expression reduces both feeding and weight gain without altering drinking or waste production. Slope of weight gain in rats injected with AAVGAD versus rats injected with placebo and controls is illustrated in Fig. 8. Examination of food consumption in the third postoperative week indicated that tolerance to the behavioral impact of GAD gene transfer may occur, however.

Gene-based neuromodulation for pain

The (CCI) model of pain was developed by Bennett and Xie [79] in 1988 to simulate human neuropathic pain in animals. The hind paws of rats are injected with solutions of capsaicin or Freund's adjuvant, inducing an inflammatory response. Application of loose ligatures to the sciatic nerve overnight induces a guarding response of the affected paw. In addition, animals keep the affected paw retracted so as not to make contact with the floor. The guarding response is a characteristic symptom in patients with RSD and causalgia. Hyperalgesia and allodynia were observed in the model and were similar to that occurring in RSD patients [79].

Use of nonviral or naked DNA gene transfer has been attempted in the CCI model. Lin et al [6] used in vivo electroporation to transfer the gene for proopiomelanocortin (POMC) encoded in naked plasmid DNA. They demonstrated that five iterations of 200 V lasting 75 milliseconds in duration given at 925-millisecond intervals yielded optimal POMC gene expression in the spinal cord. After establishing the optimal conditions for naked DNA transfer, the group evaluated the impact of POMC gene expression on the CCI model of pain. Animals that underwent POMC gene transfer had an elevated pain threshold.

Yao et al [80] used intrathecal naked DNA transfer with and without lipofectamine to deliver interleukin-2 (IL-2) to the spinal cord. The radiant heat withdrawal assay was applied to the sciatic CCI model of pain in rats. Rats treated with intrathecal recombinant IL-2 demonstrated analgesia lasting 10 to 25 minutes. The use of naked

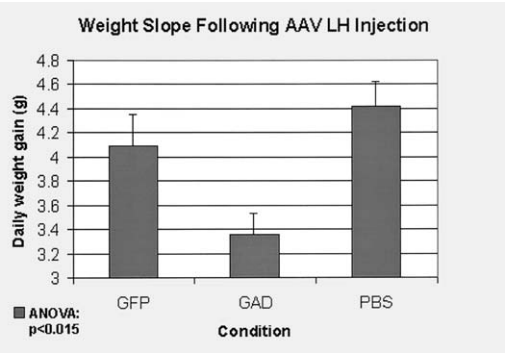

Fig. 8. Slope of average weight in rats. A postoperative comparison of the slope of weight gain in rats injected with adenoassociated virus glutamate decarboxylase (GAD) versus rats injected with placebo and controls. A significant decrease in weight gain is evident in the GAD rats. (See also Color Plate 8.)

DNA to transfer an expression cassette containing the human IL-2 cDNA under the control of the CMV promoter succeeded in producing analgesia for 6 days, with a peak effect between days 2 and 3. The intrathecal application of the IL-2 expression cassette complexed to lipofectamine induced a more profound analgesia over a similar period of time. Human IL-2 mRNA and protein were detected in the rat pia mater, DRG, sciatic nerve, and dorsal horn. No gene transfer was detected in the gastrocnemius muscle, suggesting that no transsynaptic movement of gene occurred. The gene-based IL-2 analgesia effect was blocked by opiate receptor blockade, implicating the endorphin/enkephalin system in the mechanism for this effect.

Naked DNA gene transfer to skin has been attempted as an approach to chronic pain. Lu and colleagues [81] used the gene-gun to transfer the expression cassette for POMC under the control of the CMV promoter into skin. The gene-gun employs microcarriers to deliver DNA-coated microprojectiles to target cells [82]. Control animals underwent gene-gun naked DNA transfer of a similar plasmid encoding the GFP. Immunohistochemistry revealed a threefold to fourfold elevation in the level of POMC expression in skin biopsied from experimental versus control animals 3 days after treatment. Serum levels of β-endorphin were elevated in both POMC-treated and POMC plus naloxone–treated animals compared with the GFP-expressing group. POMC gene transfer to skin was tested in the formalin model for inflammatory pain. Three days after gene-gun treatment, 1% formalin was injected subcutaneously into the region of skin that had received gene transfer. The number of paw flinches was recorded during the initial minute and for 1 minute in every 5 minutes thereafter for up to 1 hour. The phase 1 response was defined as occurring in the first 10 minutes and the phase 2 response as occurring over 10 to 60 minutes. Gene transfer had no impact on the acute nociception and phase I response to formalin. The phase II response, thought to relate to afferent neuron sensitization by formalin, was inhibited by skin expression of POMC, however. This effect was blocked by the injection of intraperitoneal naloxone, implicating opiate receptors in the mechanism and not a parallel pathway. Yao et al [83] compared the effects of naked DNA IL-2 gene transfer in skin with intrathecal delivery. Using the carrageenan-induced model of pain, this group demonstrated that intrathecal naked DNA IL-2 gene transfer was more potent and lasted longer than the effect of planter subcutaneous naked DNA gene transfer. As in this group's other published report, liposomal preparations of naked DNA outperformed raw naked DNA.

Viral vectors have also been applied to gene transfer in the subarachnoid space. Finegold et al [84] transduced the spinal pia mater with cerebrospinal fluid (CSF) administration of an adenoviral vector containing an expression cassette for the production of β-endorphin. These investigators applied pial gene transfer to an inflammation model for persistent pain. They reported reduced hyperalgesia without a change in baseline nociception.

As discussed previously, HSV vectors are inherently neurotropic. The fact that HSV demonstrates enhanced uptake into the cells of the DRG lends HSV vectors to applications aimed at pain control. Wilson et al [85] reported the construction of a replication-incompetent HSV vector containing an expression cassette with the preproenkephalin (PPE) gene under the control of the human CMV promoter. This transgene encodes five met-enkephalin molecules and one leu-enkephalin molecule. The investigators applied this vector to abraded dorsal hind paw skin in mice and found gene expression in DRG neurons with transgene expression in the dorsal horn terminals of these cells. Behavioral testing with the heat-induced foot withdrawal assay revealed no change in threshold, suggesting normal function of Aδ and C fibers. Capsaicin-induced sensitization of foot withdrawal thought to involve C fibers and dimethyl sulfoxide sensitization thought to involve Aδ fibers were both

significantly inhibited by PPE remote gene delivery, however. Both systemic and intrathecal naloxone administration reversed the effect of the PPE expression.

Goss et al [86] demonstrated that subcutaneous injection of the same vector had the ability to reduce the nociceptive impact of subcutaneous formalin injection. The tonic phase of formalin response 20 to 70 minutes after injection was most profoundly affected. This effect could be blocked by intrathecal naltrexone, implicating spinal cord opiate receptors in the mechanism of action. Gene-based enkephalin antinociception wore off over 1 month; however, surprisingly, reinjection resulted in a replacement of antinociception. Unlike HSV in this experiment, repeated peripheral administration of adenoviral vectors is not effective at replacing lost gene expression [87].

In addition to blocking limb pain, the HSV PPE vector has been applied to both interstitial cystitis and rheumatoid arthritis. Yoshimura et al [88] confirmed elevated enkephalin levels in the bladder and appropriate lumbar and sacral DRG by quantitative polymerase chain reaction (PCR) after bladder wall injection of HSV PPE. Using cystometrograms to assay bladder hyperactivity, the investigators demonstrated that HSV PPE–injected animals had a reduced response to intravesicular capsaicin compared with control animals injected with a similar vector encoding an inactive reporter gene. As with other applications of this vector, the effect was blocked by opiate receptor antagonists. Braz et al [89] constructed a similar vector with an expression cassette containing the rat PPE cDNA. Using the adjuvant-induced polyarthritis rat model for rheumatoid arthritis, this group applied remote HSV PPE delivery to DRG neuron PPE delivery. As with other studies, PPE levels in the DRG were enhanced. Rats treated with HSV PPE had not only reduced hyperalgesia but improved locomotion and reduced progression of destructive bone loss. Thus, in this model, the correction of chronic pain led to secondary improvement in the primary disease process, implying a dynamic relation between the two.

Engineered cell lines for the treatment of pain

Gene transfer can be applied to cells in vitro to affect their behavior in such a way that they can be used as tools for the treatment of pain. This approach, also referred to as ex vivo gene therapy (Fig. 9), has been applied to the P19 embryonal carcinoma cell line. The promoter for mouse mammary tumor virus (MMTV) is repressible by histone deacetylase (HDAC) inhibitors. Ishii et al [90] used a pMAMneo plasmid containing an expression cassette that used the MMTV LTR promoter to drive expression of the β-endorphin [90]. The MMTV LTR is a promoter that is able to be regulated and is sensitive to dexamethasone. Thus, the transformed P19 cells were capable of producing β-endorphin in vitro in the presence of dexamethasone. Animals that underwent subarachnoid transplantation of the engineered cell line demonstrated analgesia to both the hot plate and formalin tests for up to 1 month after transplantation. Intraperitoneal dexamethasone (100 mg/kg) amplified the analgesia by a factor of two, suggesting that the transformed P19 cells maintained dexamethasone responsiveness after implantation.

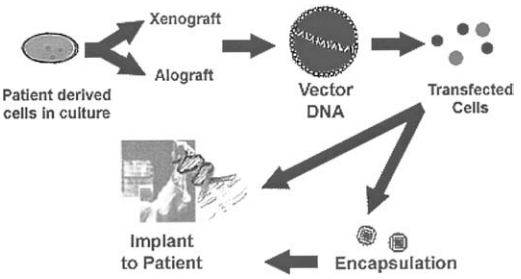

Fig. 9. Ex vivo gene transfer. The delivery of analgesic proteins can be affected through gene transfer to cell lines that are subsequently transplanted into the nervous system. Ex vivo gene therapy involves removing tissue from the patient, transfecting the cells in culture, and then reimplanting the genetically altered cells to the patient. (See also Color Plate 9.)

Eaton et al [91] transfected the RN33B neuronal cell line with naked DNA (pCEP4 plasmid) containing an expression cassette driving the production of the preprogalanin (PPG) cDNA. Galanin is a neuropeptide implicated in synaptic inhibition and observed to prolong morphine analgesia. The PPG-transfected cells (33GAL.19 cells) were shown to produce galanin immunoreactivity at low levels in dividing cells and at higher levels in terminally differentiated cells compared with a control cell line transfected with pCEP4 alone (33V.1 cells). Both cell lines survived at least 7 weeks after transplantation into the lumbar subarachnoid space. Using the sciatic CCI model of pain, 33GAL.19 animals showed analgesia to tactile and cold allodynia and thermal hyperalgesia. This effect peaked between 1 and 3 weeks. The reduction

of analgesia despite graft survival suggested either tolerance to galanin secretion or a reduction in transgene production.

Hagihara et al [92] have used the mouse neuroblastoma cell line (NTP) transformed with naked DNA containing an expression cassette for the POMC gene. The cells were xenografted into the CSF space of rats after encapsulation in a polymer capsule. In initial efforts at application of this system, secretion of corticotrophin and β-endorphin was demonstrated but proved difficult to control. For this reason, the group applied the tet-on promoter system to control gene expression in the polymer-encapsulated cells. Control of secretion was demonstrated with doxycycline application; however, continuous administration of doxycycline resulted in a loss of tet responsiveness. Tet responsiveness was maintained with intermittent doxycycline administration.

Ex vivo gene therapy has been attempted with viral vector gene transfer to primary fibroblasts. Beutler et al [93] used a retroviral vector to deliver an expression cassette for a hybrid protein. This protein was a fusion of the mouse pre-pro NGF with β-endorphin. This group found that an amino terminal signal peptide was not necessarily sufficient to induce neuropeptide secretion. The endorphin fusion protein expression cassette was, however, proven capable of secreting pharmacologically active β-endorphin. The latter was proven both with receptor displacement studies as well as with immunoreactivity. The authors proposed application of this cell line to opiate-sensitive pain states through stereotactic implantation.

Gene therapy for the root causes of pain

The vast majority of efforts at gene therapy have focused on altering the pathophysiology of individual diseases rather than on neuromodulation. Several of these diseases produce chronic pain as one of their major sequelae. Our laboratory has applied therapeutic vectors to diabetic neuropathy. Adenoviral delivery of the manganese superoxide dismutase I (MnSOD1) gene may reduce the impact of hyperglycemia on the combined motor action potential (CMAP) and latency of conduction in the rat sciatic nerve. DRG neuronal gene expression was also documented after intraneural injection of rAAV in diabetic rats, implying the potential for application of this vector system to diabetic neuropathy (Fig. 10).

Similarly, gene delivery has been proposed as a method to alleviate idiopathic nerve entrapment [94]. Gene delivery has also been proposed as a method to limit the underlying joint deterioration in chronic inflammatory joint disease [95] and rheumatoid arthritis [96,97].

Joint fusion has long been applied to the problem of mechanical low back and cervical pain. Viral vector–based delivery of the bone morphogenic protein (BMP) provides a potentially low-cost alternative to use of the expensive recombinant protein [98]. Such a vector may reduce the quantity of necessary bone grafts and risk of pseudoarthrosis with fusion. BMP delivery through adenoviral constructs may prove a useful application of first-generation adenoviral constructs. These vectors are unlikely to find application in the nervous system because of the transience of the gene expression and accompanying inflammation that they induce. In the case of interbody fusion, however, transient expression of BMP would be desirable. Further, a modicum of inflammation would be likely to increase the rate of fusion rather than posing a risk.

Dabrosin et al [99] proposed the use of angiostatin gene transfer as a means of treating chronic pelvic pain secondary to endometriosis. This group demonstrated that the established endometriosis in estrogen-supplemented ovariectomized mice could be eliminated through peritoneal administration of an adenoviral vector for angiostatin (AdAngiostatin). AdAngiostatin or control virus Ad*dl*70-3 1×10^9 in 1 mL of phosphate-buffered saline (PBS) was administered intraperitoneally into C57BL/6 mice. The positive control was partially purified rabbit angiostatin

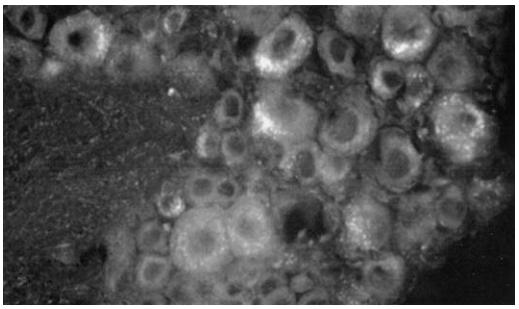

Fig. 10. Gene expression in dorsal root ganglia (DRG) after intraneural recombinant adenoassociated virus injection in diabetic rats. The presence of green fluorescent protein in DRG sensory neurons serves as a positive indication of gene expression. (See also Color Plate 10.)

derived from human urokinase-digested rabbit plasminogen. One hundred percent of the animals treated with AdAngiostatin (N = 14) experienced complete resolution of endometriosis compared with a control group of animals with a 15% remission rate (N = 13). Dabrosin et al [99] also showed that AdAngiostatin was capable of reducing sex steroid levels without inducing total castration in normally cycling animals. The group noted that the use of adenoviral gene transfer only yielded 6 to 10 days of angiostatin overexpression, however. Additionally, the use of nontargeted adenovirus created significant side effects related to impaired ovarian function.

Severe chronic pain can characterize inborn errors of metabolism. One example is Anderson-Fabry disease, which results from a deficiency in α-galactosidase A. This disorder presents with severe peripheral pain in childhood, culminating in death caused by multisystem organ failure in the fourth or fifth decade of life. Gene therapy as a field evolved initially as a means to treat genetic diseases of this sort. In other words, there is an inherent advantage in attempting to reverse a monogenic illness with gene therapy, because replacement of a single gene can reverse the disorder. Gene transfer has been proposed as a means for treating Anderson-Fabry disease [100]. As mentioned earlier, efforts are underway to apply viral gene transfer to a variety of inborn errors of metabolism, including Canavan disease [101]. A trial for the replacement of aspartylacylase (ASPA), the enzyme deficient in Canavan disease, is currently ongoing. This trial uses intraparenchymal injection of an rAAV vector encoding ASPA under the control of the NSE promoter. The vector used in this trial, like the one proposed for application to STN GAD gene transfer, incorporates the woodchuck posttranscriptional regulatory element (WPRE) sequence to increase the gene expression from individual mRNAs.

Chronic pain and hyperalgesia as a potential complication of gene-based neurorestoration

As discussed earlier in this article, gene therapy of the nervous system was initially focused on the correction of inborn errors of metabolism in the CNS. This effort branched out into the application of gene transfer to the delivery of trophic proteins. Romero and colleagues [102] injected NGFs and FGF2 into the spinal cord parenchyma 16 days after inducing dorsal root avulsion. In contrast to control vectors expressing β-galactosidase, experimental animals demonstrated axonal regeneration and functional recovery of thermal sensory function. Animals that had received an adenoviral vector for NGF (AdNGF) demonstrated aberrant sprouting of noninjured axons, however. This type of sprouting was associated with chronic pain and hyperalgesia. The authors point out that effective restorative gene transfer requires selective effects. Such effects can be achieved through the use of selective therapeutic transgenes or targeted vector systems.

Summary

Technologic advancements have made cell type–specific targeting, expression control, and safe and stable gene transfer possible. Animal research has provided increasing experience with gene transfer to the nervous system and sensory neurons in particular. Gene-based neuromodulation can be achieved through neuronal delivery of transgenes capable of altering synaptic function. Alternatively, ex vivo gene transfer can be used to create cell lines capable of secreting analgesic neuropeptides. Transplantation of these grafts and direct gene-based neuromodulation can be applied to the control of pain and the root causes of pain. These approaches combine anatomic and pharmacologic specificity. As the technology continues to improve, clinical application of cellular and molecular pain control is likely.

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